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PRINCIPAL INVESTIGATOR: Lawrence H. Thompson, Ph.D.

CONTRACTING ORGANIZATION: Lawrence Livermore National Laboratory
                           Livermore, California 94550

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Lawrence H. Thompson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Lawrence Livermore National Laboratory
Livermore, California 94550

E-Mail: Thompson14@llnl.gov

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13. ABSTRACT (Maximum 200 Words)

Understanding the DNA repair mechanisms for ionizing radiation (IR)-induced DNA damage and having prior knowledge of a patient's IR-specific repair capacity will help to determine how patients will respond to radiation therapy and to design more effective treatments. Our prior objective to construct a mutant cell line for the APE1 nuclease gene was not met despite numerous experiments and multiple approaches. Therefore, we initiated two new objectives dealing with other enzymatic cellular components that are essential for IR damage recognition and repair. First, we evaluated the new technology of siRNA knockdown of gene expression for both APE1 and polynucleotide kinase (PNK), a protein that plays a major role in processing the termini of DNA breaks. The APE1 experiments were unsuccessful, for unknown reasons, but experiments with PNK look promising based prior experience with human tumor cells. Second and most important, we have shown that we can use immuno-fluorescence to detect the sites of double-strand breaks (DSBs) caused by IR in immortalized normal human fibroblasts. The assay is extremely sensitive, with low background, and is linear with dose from 0 to 90 cGy. This immuno-detection system has as wide application to studying the mechanisms of DSB repair and will be used extensively in future studies.

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Project Title: Repair Machinery for Radiation-Induced DNA Damage

Principal Investigator:
Lawrence H. Thompson (initially David M. Wilson III)
Biology & Biotechnology Research Program, L-441
Lawrence Livermore National Laboratory
Regents of the University of California
7000 East Avenue
Livermore, CA 94551-0808
Office: (925) 422 5658
Fax: (925) 422 2099
Thompson14@llnl.gov

INTRODUCTION

Current methods to eradicate breast cancer and preserve normal tissue involve total mastectomy followed by radiotherapy. However, the use of postoperative radiotherapy has produced mixed results. While some randomized studies indicate that there is no major benefit from post-mastectomy radiation treatment, there exists a subgroup of patients that benefit from this type of strategy. Thus, it is imperative that we design effective prognostic tools to identify patients who will profit, as well as those who may be harmed, by adjuvant radiotherapy. Ionizing radiation (IR) is cytotoxic to cells largely because it introduces lethal genetic damage in the form of DNA strand breaks and clustered base damage. Thus, understanding the repair mechanisms for such DNA alterations and having prior knowledge of a patient's radiation-specific repair capacity will help design more effective treatment regimes and help determine which patients will be most responsive to radiation exposure. We intend to identify and characterize proteins involved in correcting the most abundant radiation-induced DNA damages. Such studies will provide the necessary tools (both specific DNA repair genes and biochemical assays) for predicting an individual's repair capacity and thus potential IR sensitivity (genetic predisposition).

BODY

Objective 1: To construct an *APE1* gene knockout cell line and examine the role of Ape1 protein in DNA repair

Background. Radiation induces an array of DNA damages, including abasic lesions and strand breaks harboring 3′-blocking termini such as phosphoglycolate and phosphate groups (Ward, 1988; Ward, 1995; Ward, 1998). The human Ape1 protein has been shown to incise at AP sites and remove a subset of 3′-damages, as well as to stimulate the DNA-binding activity of several oncoproteins (e.g. p53, Fos and Jun) in vitro (Jayaraman et al., 1997; Suh et al., 1997; Wilson et al., 1995; Xanthoudakis et al., 1992). Yet despite the basic understanding of

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the biochemical properties of Ape1, the in vivo function of the protein remains largely unclear, particularly as it relates to IR protection. The production of *APE1* knockout cell lines represents an important step towards defining the biological contribution of this mammalian protein.

Attempts to improve targeting efficiency of the *APE1* gene. As described in detail in our last report, there were multiple difficulties associated with gene targeting experiments for this particular gene (although we have had success with three others in other projects). We used very large numbers of cells to compensate for the low targeting efficiency, combined with stringent and tedious diagnostic testing to avoid false positives due to non-targeted random integration of the vector. Therefore, we decided to employ the *APRT* gene as a model targeting system in the A2tg CHO cells and test conditions that might improve targeting efficiency by, for example, reducing random integration of the targeting vector.

Employment of A2tg CHO cells for *APRT* targeting. A2tg cells (derived from our standard AA8 cells) are physically hemizygous at the *APRT* locus, with an inactivating point mutation at the remaining allele. The targeting vector, pGS73, contains the *APRT* promoter and part of the coding sequence (2.3 kb) of the gene. Recombination between the *APRT* locus and the targeting vector is necessary for acquiring a functional *APRT* gene, allowing cells to grow in ALASA selective medium.

Testing cellular treatments to enhance gene targeting. The A2tg model system was used to assess treatments of the cells before transfection that might enhance gene targeting efficiency. Cellular treatments tested included cell cycle arrest by excess thymidine and transient transfection of expression plasmids for the mismatch repair gene *MSH3*, as well as *RAD54B*. Treatment of cells with thymidine arrests cells in the S phase of the cells cycle. In addition, cells require the activity of homologous recombination (HR) repair to recover from this arrest of DNA replication, suggesting a means of stimulating HR in a way that might promote gene targeting (Lundin et al., 2002). Since gene targeting is thought to be most efficient during S-phase when free DNA ends are available during lagging strand synthesis, release of thymidine arrested cells at the G1/S boundary before transfection of the targeting vector was predicted to enhance the targeting efficiency.

The DNA mismatch repair (MMR) system stringently prevents recombination from occurring, particularly between slightly mismatched sequences (de Wind et al., 1995). Overexpression of the MMR protein MSH3 is known to reduce the efficiency of the MMR system (Marra et al., 1998), possibly allowing for an increase in homology-driven recombination and a tolerance for minor heterology that could exist between the genomic DNA and the targeting vector. Finally, since inactivation of *RAD54B* was reported to reduce targeted integration in previous studies in human cells (Miyagawa et al., 2002), we thought that its overexpression might enhance targeting.

In our experiments, thymidine arrest prior to electroporation reduced the overall transfection efficiency about 4-fold and the targeting efficiency at the *APRT* locus by only about 3-fold, providing only a slightly improved targeting efficiency. *MSH3* and *RAD54B* treatments both reduced the transfection efficiency by about 40%. *MSH3* also reduced the targeting efficiency to a similar level, while *RAD54B* only slightly lowered the targeting efficiency. None of these changes was considered of sufficient magnitude to warrant applying to the *APE1* gene.

Testing vector modifications to enhance gene targeting. Modification of the targeting vector is another strategy for possible enhancement of gene targeting efficiency. During the process of HR repair, the 5′ ends of a double-strand break are digested by nuclease, leaving long 3′ overhanging tails that interact with RPA and Rad51 to form a nucleoprotein filament, which initiates the recombination process with homologous sequence. To mimic this resectioning, in the hopes of driving the vector DNA towards a homologous integration at the targeting site, we pre-treated the linearized targeting vector with the 5′ to 3′ single-strand exonuclease T7. After, testing multiple conditions involving positions of vector linearization and exonuclease treatment time, we found that exonuclease treatment actually hindered the targeting of *APRT* in the A2tg cells. There was an overall reduction in transfection in the exonuclease-treated samples by 30 to 50%, which may be due to end degradation of the DNA after introduction into the cells. Such degradation would reduce the length of the region of homology in the targeting vectors, thereby reducing targeting efficiency. This idea was consistent with the results from these experiments since the targeting efficiency was reduced by an additional 50% from exonuclease treatment. Overall, these results indicate that pre-treatment of the targeting vector with exonuclease is not effective in enhancing gene targeting efficiency.

PARP inhibition as a possible means of improving gene targeting. PARP (Poly-ADP ribose polymerase is an enzyme that plays an important role in the recognition and repair of single-strand breaks (Fernet et al., 2000; Herceg and Wang, 2001; Jilani et al., 1999). The PARP inhibitor, 3-methoxybenzamide (3-MB), was reported in a previous study of CHO cells to be useful in reducing random integration of a targeting vector (Waldman et al., 1996). This report noted that the absolute targeting efficiency was reduced by 2-fold, but the non-specific integration was reduced between 14- and 200-fold, providing a 7-to 100-fold enrichment in targeting vs. non-specific vector integration. This modification appeared to be a very promising way of improving our *APE1* targeting efficiency, and we evaluated this approach in our standard (AA8-hAPE1-3) CHO cells using the LARA1 targeting vector described last year (*APE1* targeting vector with the gene disrupted with the puromycin-resistance marker and *HsvTK* negative selection marker). We used calcium-phosphate transfection with and without treatment of the cells with 3-MB. While we did not expect to identify an actual targeting event in these experiments (due to limitations of sample size), we measured the transfection efficiency by counting the number of puromycin-resistant colonies arising after transfection in each condition. The results, which are summarized in Table 1, were disappointing. Treatment of cells with 3-MB did not change the frequency of puromycin-resistant colonies. Without a marked reduction in the

frequency of puromycin resistant colonies, indicating inhibition of non-specific integration, such a treatment procedure is not useful, and was therefore no longer a consideration for this lab. The published results could not be confirmed.

Table 1. Effect of 3-MB treatment on transfection efficiency

Experiment	3-MB treatment	Total DNA transfected	LARA1 transfection efficiency (Puro^R)	FIAU enrichment
1	0 mM	3 μg	4×10^{-4}	2.1 ×
2	3 mM	3 μg	5×10^{-4}	1.6 ×
3	0 mM	6 μg	2×10^{-4}	1.6 ×
4	3 mM	6 μg	2×10^{-4}	1.6 ×

Objective 2 (New): To employ siRNA knockdown of gene expression to produce mutant phenotypes

Introduction to siRNA (small inhibitory RNA). A newer technology has emerged recently that may help answer questions that depend on creating protein deficiencies in cells without the need for producing gene knockouts. This is the methodology of siRNA, which takes advantage of cellular machinery to target and degrade specific transcripts, substantially reducing, if not removing entirely, the levels of the targeted mRNA and protein (Denli and Hannon, 2003; Dillin, 2003; Miller and Grollman, 2003).

APE1 siRNA. Considering the difficulties associated with making the *APE1* knockout, we decided it was desirable to employ siRNA technology to reduce APE1 level in CHO cells. Using the pSilencer™ system designed and provided by Ambion, we made vectors that, when transfected into cells, will express interfering RNA with sequence homology to CHO hamster *APE1*. Four siRNAs were tested, spaced along the *APE1* sequence, and each was transfected into AA8 cells for stable expression. Since the pSilencer™ vector does not contain a selection marker, it was co-transfected with another plasmid, pcDNA3, containing the neomycin resistance marker. After transfection, cells were plated in the presence of G418, and eight clones were picked from transfections of each construct. The lack of availability of an APE1 antibody that will detect the hamster protein necessitated measuring APE1 endonuclease activity to quantify the amount of APE1 protein in each clone. This procedure was performed by collaborator Dr. David M. Wilson (at NAI, Baltimore, MD and previous PI of this project). None of the 32 clones tested

had a significant decrease in endonuclease activity, suggesting no reduction of protein levels in any of the clones. We were surprised by the uniformly negative results since there was a report in the literature of successfully inhibiting human APE1 in differentiated cells (Fan et al., 2003).

PNK siRNA. Polynucleotide kinase (PNK) is protein that plays an important role in processing DNA strand breaks. It phosphorylates the 5′ ends of DNA OH groups and removes phosphate moieties from 3′ ends, functions that are essential for eliminating single-strand breaks produced by radiation and endonuclease action (Jilani et al., 1999). Our collaborator Dr. Michael Weinfeld successfully employed siRNA to reduce PNK in human tumor cell line using a previously reported vector (Brummelkamp et al., 2002) that contains a neomycin resistance marker, allowing for selection of cells maintaining the expression plasmid. Our goal is to create a normal (non-tumor) human fibroblast cell line that stably expresses PNK siRNA. Thus, we transfected NHF1 cells (which are discussed in more detail below) and are currently growing them in the presence of G418 to recover stable transfectants. Western analysis will be performed on G418 resistant clones to determine PNK protein levels. Further studies, such as sensitivity to damaging agents, radiation induced mutation rates, and γH2AX foci studies (also described below) will be performed on siRNA-expressing clones to correlate lack of PNK with cell phenotypes in radiation responses. We expect to have success with this study, which should lead to a publication.

Objective 3 (New): To develop an assay to detect the formation of double-strand breaks in immortalized human cells.

Background. One of the most deleterious types of DNA damage from ionizing radiation is the double-strand break (DSB), which is the lesion generally considered to be responsible for cell killing. DSBs are produced directly as frank breaks but may also arise when the DNA replication machinery encounters oxidized base damage, including clusters of lesions often referred to as multiply damaged sites. At the clinical dose of 2 Gy that is often used in fractionated radiation therapy, a cell receives an average of ~70 DSBs (Rothkamm and Löbrich, 2003), which exceeds usually exceeds it repair capacity. Incomplete repair results in 50% or more of the cells being killed. A better understanding of the mechanisms of DSB repair may provide opportunities to improve radiation therapy by blocking repair in tumor cells through the use of inhibitors of the repair systems. Also, the enzymatic repair of DSBs is strongly dependent on efficient recognition and signaling processes that recruit the repair proteins to the sites of DSBs. This mechanism of increasing the local concentration of repair proteins at the sites of damage is likely responsible for the high efficiency of DSB repair. During the last several years, several key phosphorylation events that occur almost immediately after irradiation have been identified. The first is activation of the master regulator protein ATM (Bakkenist and Kastan, 2003), which is defective in radiation-sensitive people who have the disease ataxia telangiectasia. Work during the last year has shown that only one or a couple of DSBs per cell is sufficient to activate a portion of the

ATM sensor molecules, which occurs when they undergo autophosphorylation in the vicinity of DSBs (Bakkenist and Kastan, 2003). Once ATM is activated, it exhibits kinase activity for numerous proteins that implement signaling reactions to recruit the repair and cell cycle checkpoint proteins [as we recently reviewed in (Thompson and Limoli, 2003)]. One of the first proteins to become phosphorylated is histone H2AX, which is designated γH2AX after phosphorylation (with γ signifying gamma radiation). DSBs are both necessary and sufficient for γH2AX formation (Pilch et al., 2003; Sedelnikova et al., 2002). Most important, the sites of DSBs can be detected using an antibody that specifically recognizes γH2AX, and not H2AX, by detecting the phosphate group that is transferred to Ser139 on the H2AX molecule. Moreover, some of the commercial antibodies are specific enough to detect DSBs with high efficiency and minimal background.

Efficient detection of DSBs in immortalized human cells. Our goal is to develop the use of immortalized diploid human fibroblasts as an experimental system to study molecular mechanisms of DSB repair. Recently, we acquired telomerase-immortalized human cells (line NHF1 from Dr. Bill Kaufmann, Univ. North Carolina). The key virtues of these cells are that they are chromosomally normal (diploid) and can be grown continuously in culture, allowing for standard genetic analysis based on cloning and subcloning without loss of proliferative capacity.

After testing γH2AX antibodies from two companies, we found one of them gave highly specific staining for DSBs produced by γ-irradiation in the low dose range below 1 Gy as shown in Figure 1. Since it is known that DSBs arise during DNA replication, these experiments were done using NHF1 cells that were in a high-density resting state with very few dividing cells (Go phase). The γH2AX foci are quite distinct although in the pictures some foci in a given cell are not in focus. Foci were counted by focusing up and down within each nucleus. The dose response curve for 0 to 90 cGy is shown in Figure 2. The yield of foci in this experiment is about one half the expected number based generally accepted values of the yield of DSBs per Gy, for example, as determined by (Rothkamm and Löbrich, 2003). This discrepancy indicates that we may need to vary the staining conditions to see whether we can detect a higher yield of foci. However, it is also possible the number of IR-induced DSBs per cell may vary among cell lines because of differences in nuclear geometry, chromatin packing, and other variables.

These results provide a strong foundation for future studies that will be funded in new grant applications that are being submitted. We will be able to automate the scoring with a confocal microscope facility that is now being set up on our Program. We expect to have the capability of performing "optical sectioning" through each cell and integrating the signal of each section.

KEY RESEARCH ACCOMPLISHMENTS (during the last year)
- Manuscript published on the characterization of APE1-overexpressing cells
- Initiated experiments to knockdown PNK (polynucleotide kinase) activity in immortalized diploid human cells using an expression vector that has been validated in a human tumor line by one of our collaborators.

REPORTABLE OUTCOMES
Schild, L. J., K. W. Brookman, L. H. Thompson, and D. M. Wilson III. 2002. Ape1 as a factor in cellular resistance to DNA-damaging and anti-cancer agents. Somat. Cell Mol. Genet. **25**:253-262.

CONCLUSIONS
Understanding the repair mechanisms for IR-induced DNA damage and having prior knowledge of a patient's radiation-specific repair capacity will help determine which patients will be most responsive to radiation therapy and design more effective treatment regimes. During the last year we adopted several new experimental approaches toward understanding cellular sensitivity to ionizing radiation. Unfortunately, we did not succeed in creating a mutant cell line for the *APE1* gene, but two new objectives were pursued and these provide a basis for future studies in new projects. Our ongoing experiments using stable siRNA suppression of the mRNA of the PNK gene in immortalized diploid human cells are promising since this vector appears to function correctly in human tumor cells. Our results showing quantitative detection of DSBs in human cells provides a framework for many future studies with this new system in which parameters influencing the efficiency of repair will be studied.

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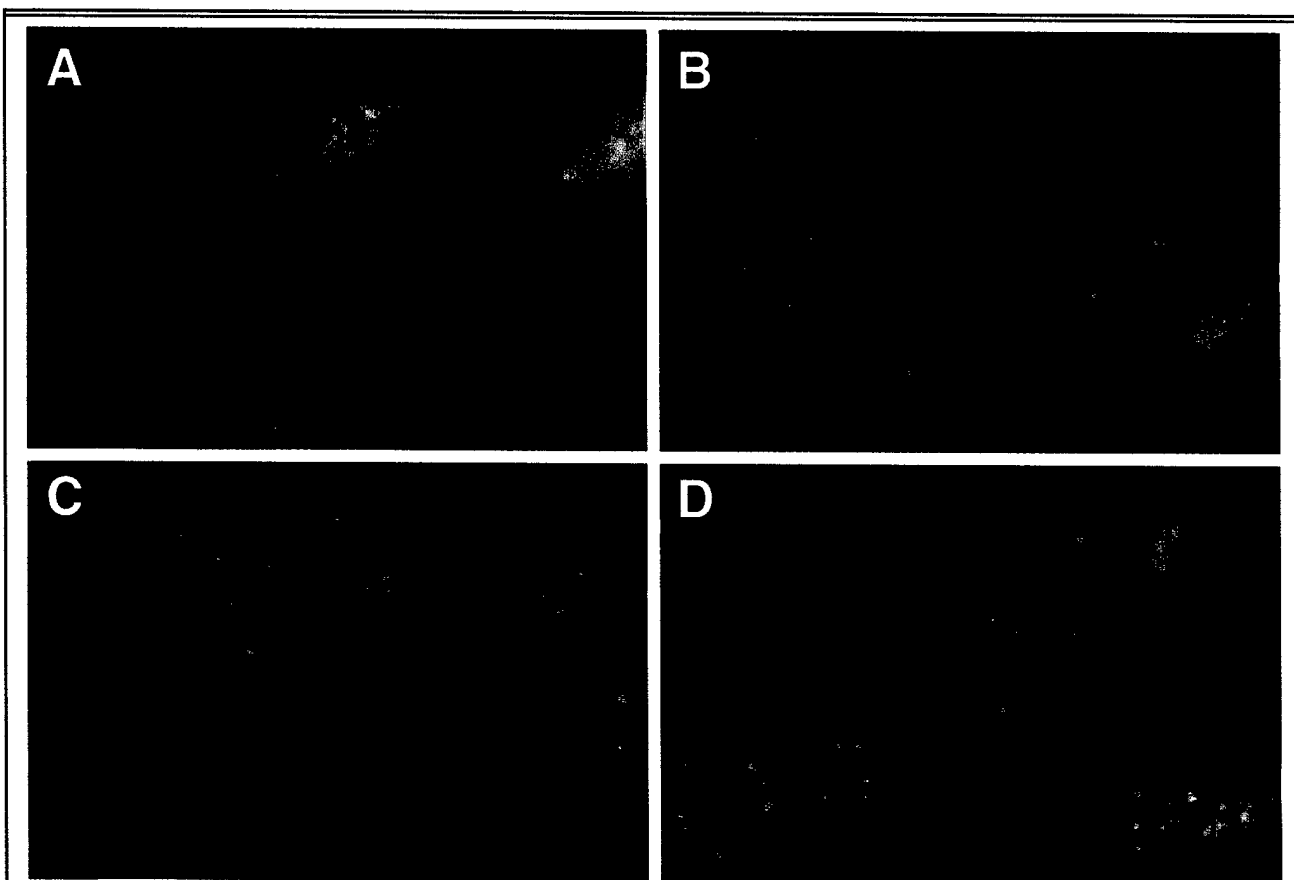
Figure 1. Immortalized human diploid fibroblasts irradiated with ^{137}Cs γ-rays and stained for γH2AX nuclear foci as a marker for DSB production. Only the nuclei of the cells are visible from DAPI staining. Cells were allowed to reach confluence in Go so that very few of the cells were cycling and replicating their DNA. Cells were irradiated, incubated at 37°C for 20 min to allow the enzymatic phosphorylation of histone H2AS to occur, and then fixed and stained with γH2AX antibody (Upstate Biologicals). Panel A, unirradiated; panel B, 10 cGy, panel C, 30 cGy, and panel D, 90 cGy.
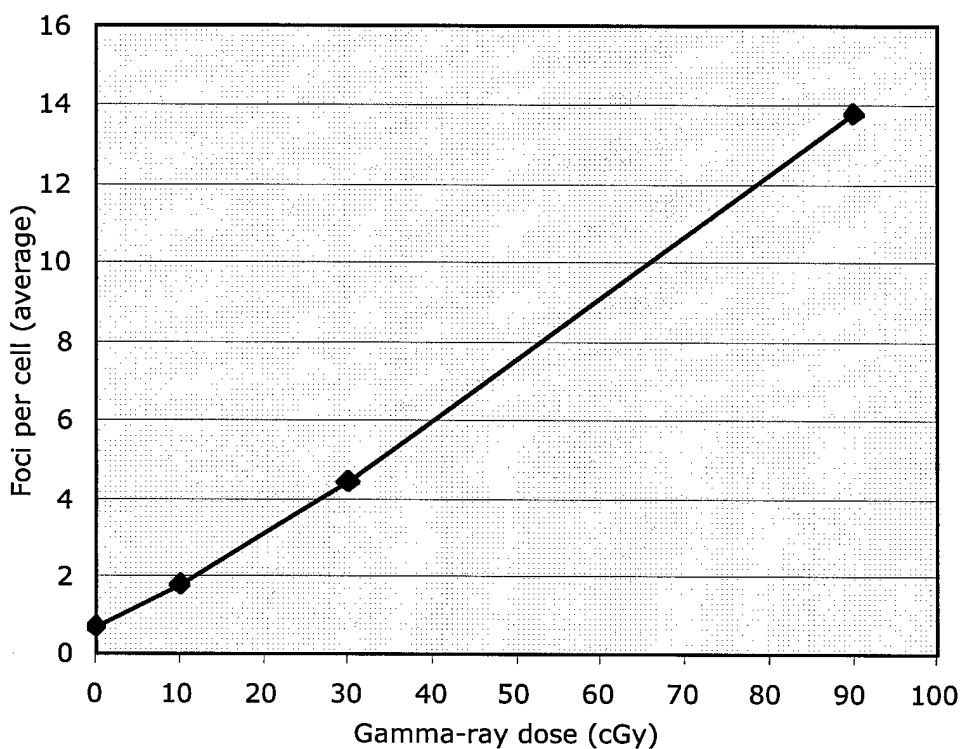
Figure 2. Number of γH2AX foci per cell as a function of γ-ray dose. Unirradiated cells have less than one focus per cell. When the background at zero dose is subtracted, the data points are a good fit to a straight line.

PERSONNEL

Role on Project	Name	Degree	Scientific Discipline	Institutional Affiliation
PI	Lawrence H. Thompson	Ph.D.	Radiation Biology & Molecular Genetics	Lawrence Livermore National Laboratory, Univ. of California (LLNL-UC)
Term staff	Robert Tebbs	Ph.D.	Molecular & Cellular Biology	LLNL-UC
Post-doctoral Scientist	John Hinz	Ph.D.	Molecular & Cellular Biology	LLNL-UC
Consultant	David Wilson (previous PI)	Ph.D.	Protein Biochemisty	National Institute on Aging (Baltimore, MD)